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ANALOGUES OF CTL EPITOPES WITH IMPROVED MHC CLASS-I BINDING CAPACITY ELICIT ANTI-MELANOMA CTL RECOGNIZING THE WILD-TYPE EPITOPE

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The MHC class-I binding affinity of an epitope is an important parameter determining the immunogenicity of the peptide-MHC complex. In order to improve the immunogenicity of an epitope derived from melanocyte lineage-specific antigen gp100, we performed amino-acid substitutions within the epitope and assayed both HLA-A*0201 binding and CTL recognition. Anchor replacements towards the HLA-A*0201 peptide-binding motif gave rise to peptides with higher HLA-A*0201 binding capacity compared to the wild-type epitope. In addition, several of the gp100 154–162 epitope-analogues were more efficient at target-cell sensitization for lysis by anti-gp100 154–162 CTL compared to the wild-type epitope. These altered gp100 154–162 epitopes were subsequently tested for their capacity to induce CTL responses *in vivo* using HLA-A*0201/K^b transgenic mice, and *in vitro* using HLA-A*0201⁺ donor-derived lymphocytes. Interestingly, the peptide-specific CTL obtained, which were raised against the different gp100 154–162 epitope-analogues, displayed cross-reactivity with target cells endogenously processing and presenting the native epitope. These data demonstrate that altered epitopes can be exploited to elicit native epitope-reactive CTL. The use of epitope-analogues with improved immunogenicity may contribute to the development of CTL-epitope based vaccines in viral disease and cancer. *Int. J. Cancer*, 70:302–309, 1997.

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Cytotoxic T cells recognize short peptide fragments of 9–11 amino acids in length, which are presented in the antigen-binding groove of MHC class-I molecules. These peptides are usually derived from intracellular protein pools and associate in the lumen of the endoplasmic reticulum with MHC class-I heavy-chain and β_2 -microglobulin molecules, after which the MHC-peptide complex is transported to the cell surface. Despite the presence of many putative antigenic peptides within the same antigen, only a few peptides are selected for recognition by CTL.

A major parameter determining cell-surface presentation of a given peptide is the affinity of the peptide for MHC class-I molecules. Analysis of HLA-A*0201-restricted epitopes recognized by anti-viral CTL demonstrated that the majority of these peptides bind to HLA-A*0201 with high affinity. Furthermore, immunogenicity analysis of motifs containing potential epitopes, using HLA-A*0201 transgenic mice, revealed that a threshold MHC class-I affinity was required for a peptide to elicit a CTL response (Sette *et al.*, 1994). In addition to the MHC class-I binding affinity, stability of peptide-MHC complexes at the cell surface contributes to the immunogenicity of a CTL epitope (van der Burg *et al.*, 1996). Consequently, MHC class-I binding affinity and stability of peptide-MHC complexes are important criteria in the selection of specific peptide determinants for development of CTL-epitope-based therapeutic vaccines.

Recently, a number of antigens have been identified as targets for anti-melanoma CTL. Using a genetic approach, Boon and colleagues identified the tumor-specific antigens MAGE-1 and -3 as well as the melanocyte-lineage-specific antigen tyrosinase (Van den Eynde and Brichard, 1995). Similarly, we identified the gp100 melanocyte-specific protein as a target antigen for melanoma tumor-infiltrating lymphocytes (Bakker *et al.*, 1994). Recently, 2 other melanocyte differentiation antigens, Melan-A/MART-1 and

gp75, were identified as target antigens for anti-melanoma CTL (Van den Eynde and Brichard, 1995). Eight HLA-A*0201 restricted epitopes derived from these antigens have now been characterized, displaying varying affinities for HLA-A*0201 (Kawakami *et al.*, 1995; Bakker *et al.*, 1995b; Van den Eynde and Brichard, 1995).

In the present study, we closely examined one of the HLA-A*0201 presented epitopes derived from the melanocyte differentiation antigen gp100 that does not strictly follow the common HLA-A*0201 peptide-binding motifs. In an attempt to improve the immunogenicity of the gp100 154–162 epitope, we performed amino-acid substitutions within the epitope to increase HLA-A*0201 binding capacity. We demonstrate that epitope analogues allow the induction of peptide-specific CTL displaying cross-reactivity with target cells endogenously processing and presenting the native epitope. Usage of epitope analogues with improved immunogenicity may contribute to the development of CTL-epitope based vaccines in chronic viral disease and cancer.

MATERIAL AND METHODS

Cell culture

The HLA-A*0201⁺ melanoma line BLM was cultured as described previously (Bakker *et al.*, 1994). TIL 1200 lymphocytes and the melanoma lines Mel 624 and Mel 397 were a kind gift from Dr. Y. Kawakami (National Cancer Institute, National Institutes of Health, Bethesda, MD) and were cultured as reported previously (Kawakami *et al.*, 1992). T2 cells and HLA-A*0201⁺ B lymphoblastoid JY cells were maintained in Iscove's medium (GIBCO, Paisley, UK) supplemented with 5% FCS (BioWhittaker, Verviers, Belgium). Jurkat A*0201/K^b cells expressing the HLA-A*0201/K^b chimeric molecule were cultured in Iscove's medium with 5% FCS supplemented with 0.8 mg/ml G418 (GIBCO).

HLA-A*0201⁺ lymphocytes

Healthy Caucasian volunteers were HLA-A2 phenotyped by flow cytometry using MAbs BB7.2 and MA2.1. The donors underwent leukapheresis, and PBMC were isolated by Ficoll/Hypaque density gradient centrifugation. The cells were cryopreserved in aliquots of 4×10^7 PBMC.

Transgenic mice

HLA-A*0201/K^b transgenic mice were kindly provided by Dr. L. Sherman (San Diego, CA, through Harlan Sprague Dawley,

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Indianapolis, IN). Mice were kept under clean conventional conditions. The transgenic mice express the product of the HLA-A*0201/K^b chimeric gene in which the α_3 domain of the heavy chain is replaced by the corresponding murine H-2 K^b domain while leaving the HLA-A*0201 α_1 and α_2 domains unaffected (Vitiello *et al.*, 1991). This allows the murine CD8 molecule on the murine CD8⁺ T lymphocytes to interact with the syngeneic α_3 domain of the hybrid MHC class-I molecule.

Peptides

For induction of CTL and chromium-release assays, peptides were synthesized with a free carboxy-terminus by Fmoc peptide chemistry using an ABIMED multiple synthesizer. All peptides were >90% pure as indicated by analytical HPLC. Peptides were dissolved in DMSO and stored at -20°C.

Competition-based HLA-A*0201 peptide-binding assay

Peptide binding to HLA-A*0201 was analyzed using HLA-A*0201⁺ JY cells as described previously (van der Burg *et al.*, 1995). Briefly, mild-acid-treated JY cells were incubated with 150 nM fluorescein (FL)-labelled reference peptide [FLPSDC(-FL)FPSV] and with several concentrations of competitor peptide for 24 hr at 4°C in the presence of 1.0 µg/ml β_2 -microglobulin (Sigma, St. Louis, MO). Subsequently, the cells were washed, fixed with paraformaldehyde and analyzed by flow cytometry. The mean fluorescence (MF) obtained in the absence of competitor peptide was regarded as maximal binding and equated to 0%; the MF obtained without reference peptide was equated to 100% inhibition. The percentage inhibition of binding was calculated using the formula:

$$\frac{[1-(MF \text{ 150 nM reference and competitor peptide} - MF \text{ no reference peptide})]}{(MF \text{ 150 nM reference peptide} - MF \text{ no reference peptide})} \times 100\%.$$

The binding capacity of competitor peptides is expressed as the concentration needed to inhibit 50% of binding of the FL-labelled reference peptide (IC₅₀).

Measurement of MHC-peptide complex stability at 37°C

Measurement of MHC-peptide complex stability was performed as previously described (van der Burg *et al.*, 1996). HLA-A*0201⁺ homozygous JY cells were treated with 10⁻⁴ M emetine (Sigma) for 1 hr at 37°C to stop *de novo* synthesis of MHC class-I molecules. The cells were then mild-acid-treated and subsequently loaded with 200 µM of peptide for 1 hr at room temperature. Thereafter, the cells were washed twice to remove free peptide and incubated at 37°C for 0, 2, 4 and 6 hr. Subsequently, the cells were stained using MAb BB7.2 (Parham and Brodsky, 1981), fixed with paraformaldehyde and analyzed by flow cytometry.

CTL induction in HLA-A*0201/K^b transgenic mice

Groups of 3 HLA-A*0201/K^b transgenic mice were injected subcutaneously at the base of the tail vein with 100 µg peptide emulsified in IFA in the presence of 140 µg of the H-2 I-A^b-restricted HBV core-antigen-derived T-helper epitope (128–140; sequence TPPAYRPPNAPIL). After 11 days, mice were killed and spleen cells (30 × 10⁶ cells in 10 ml) were restimulated *in vitro* with syngeneic irradiated LPS-stimulated B-cell lymphoblasts pulsed with the peptide used for immunization (ratio 4:1). At day 6 of culture, the bulk responder populations were tested for specific lytic activity.

HLA-A*0201⁺ donor-derived CTL induction in vitro

Using thawed PBMC, we generated dendritic cells according the procedure of Romani *et al.* (1994) as described (Bakker *et al.*, 1995a). Before the beginning of culture, dendritic cells were loaded with 50 µM of peptide. Autologous CD8⁺-enriched responder T lymphocytes were prepared by allowing thawed PBMC to adhere

for 2 hr and by subsequent partial depletion of the non-adherent fraction of CD4⁺ T cells using the anti-CD4 MAb RIV-7 and sheep anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Sweden). At the beginning of stimulation, 2 × 10⁵ peptide-loaded DC and 2 × 10⁶ responder cells were co-cultured per well of a 24-well tissue-culture plate (Costar, Badhoevedorp, The Netherlands) in 2 ml of Iscove's medium containing 5% pooled human AB⁺ serum, 10³ U/ml IL-6 (Sandoz, Basel, Switzerland) and 5 ng/ml IL-12 (kindly provided by Dr. M. Gately, Hoffmann-La Roche, Nutley, NJ).

On day 8 and day 15, the responder populations were restimulated using peptide-pulsed dendritic cells as stimulator cells. The cultures were propagated in medium containing IL-2 (Cetus, Emeryville, CA) and IL-7 (Genzyme, Cambridge, MA) at final concentrations of 10 U/ml and 5 ng/ml respectively. At weekly intervals thereafter the cultures were restimulated using adherent-peptide-pulsed PBMC as described (Bakker *et al.*, 1995a). Responder populations were tested for specific lytic activity after at least 4 rounds of restimulation.

Chromium-release assay

Chromium release assays were performed as described (Bakker *et al.*, 1994). Briefly, 10⁶ target cells were incubated with 100 µCi Na₂⁵¹CrO₄ (Amersham, Aylesbury, UK) for 1 hr. Various amounts of effector cells were then added to the target cells in triplicate wells of U-bottomed microtiter plates (Costar) in a final volume of 150 µl. In peptide recognition assays, target cells were pre-incubated with various concentrations of peptide for 30 or 60 min at 37°C in a volume of 100 µl prior to the addition of effector cells. After 5 hr of incubation, part of the supernatant was harvested and its radioactive content was measured. The mean percentage specific lysis of triplicate wells was calculated using the formula:

$$\% \text{ specific lysis} = \frac{[(\text{experimental release} - \text{spontaneous release})]}{(\text{maximal release} - \text{spontaneous release})} \times 100$$

RESULTS

Identification of amino-acid residues engaged in HLA-A*0201 binding and/or TCR interactions for the gp100 154–162 epitope

The gp100 154–162 epitope has been identified by means of an HLA-A*0201-restricted TIL line derived from a metastatic melanoma. Among a panel of peptides ranging from 8-mers to 11-mers located around gp100 amino acids 155–161, we identified the

TABLE I – HLA-A*0201 BINDING AND TARGET-CELL SENSITIZATION OF ALANINE-REPLACEMENT EPIOTOPES

gp100 154–162	Affinity IC ₅₀ (µM) ¹	Target-cell lysis by TIL 1200 ²
KTWGQYWQV	1.4	67
ATWGQYWQV	1.3	75
KAWGQYWQV	1.5	64
KTAGQYWQV	6.2	58
KTWAQYWQV	1.6	63
KTWGAYWQV	1.4	9
KTWGQAWQV	0.9	7
KTWGQYAV	4.3	-2
KTWGQYWAV	0.5	73
KTWGQYWQA	1.9	76

¹Binding of peptides to HLA-A*0201 was analyzed in a competition assay at 4°C using mild-acid-treated HLA-A*0201⁺ B-LCL. The binding capacity of the peptides is shown as the concentration of peptide needed to inhibit 50% of binding of the fluorescein-labelled reference peptide. The affinity of the control HBC core antigen 18–27 peptide is 0.5 µM. ²Numbers indicate % specific lysis by the relevant TIL lines at an E:T ratio of 20:1. Chromium-labelled T2 target cells were pre-incubated for 90 min with 1 µM of peptide. Chromium release was measured after 5 hr of incubation. Control T2 cells loaded with an irrelevant HLA-A*0201 binding peptide were not lysed (3%).

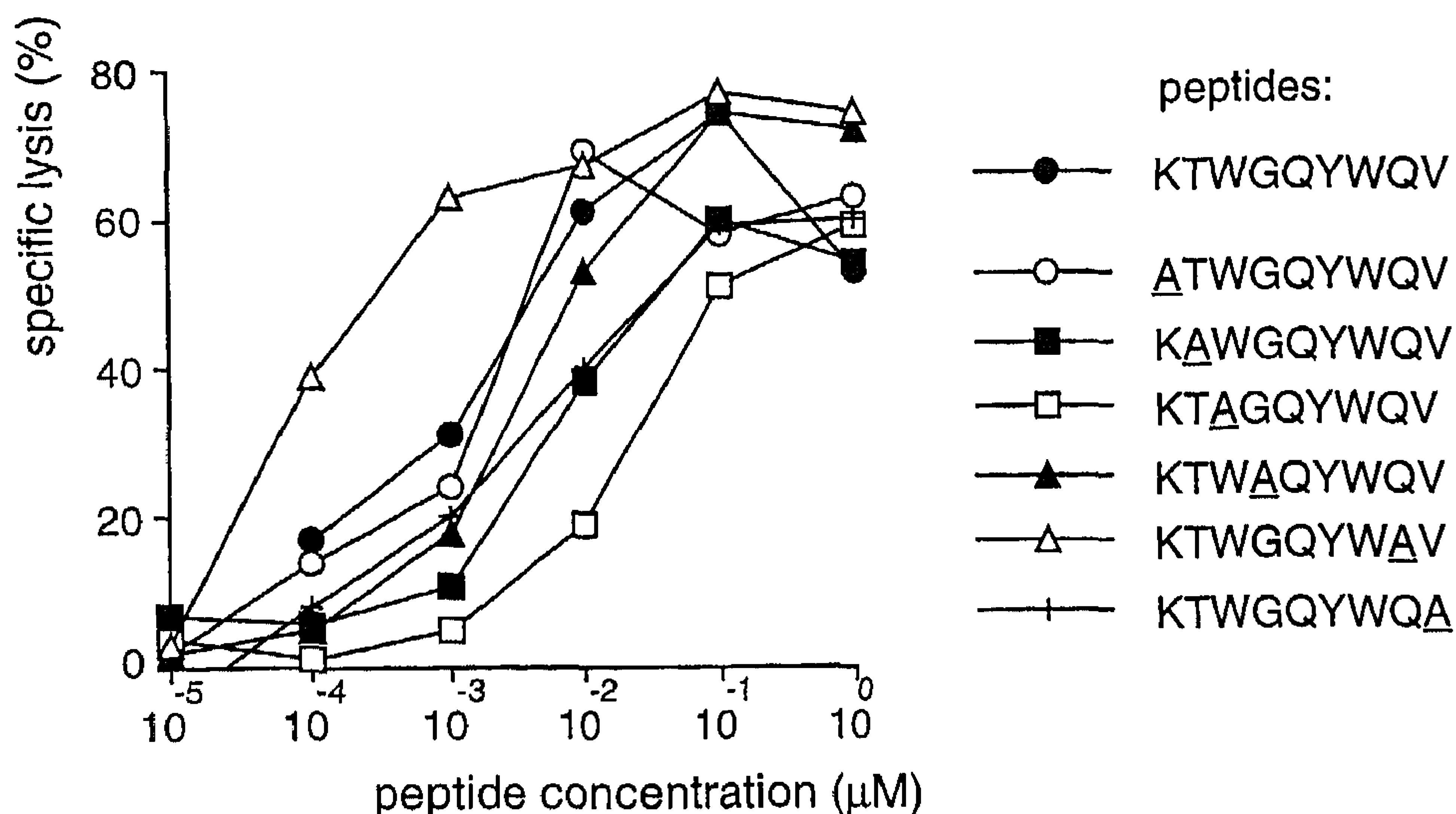


FIGURE 1 – Target-cell sensitization of gp100 154–162 alanine-replacement epitopes. Chromium-labelled T2 target cells were pre-incubated for 1 hr with various amounts of the indicated alanine-substituted epitope analogues. Gp100 154–162-reactive TIL 1200 lymphocytes were added at an effector-to-target ratio of 20:1.

TABLE II – HLA-A*0201 BINDING AND TARGET CELL SENSITIZATION OF N-TERMINAL ANCHOR-REPLACEMENT EPITOPES

gp100 154–162	Affinity IC ₅₀ (μM) ¹	Target-cell lysis by TIL 1200 ²
KTWGQYWQV	1.4	67
KVWGQYWQV	0.8	69
KLWGQYWQV	0.4	65
KMWGQYWQV	0.6	57
KIWGQYWQV	0.6	68

¹Binding of peptides to HLA-A*0201 was analyzed in a competition assay at 4°C using mild-acid-treated HLA-A*0201⁺ B-LCL. The binding capacity of the peptides is shown as the concentration of peptide needed to inhibit 50% of binding of the fluorescein-labelled reference peptide. ²Numbers indicate % specific lysis by the relevant TIL lines at an E:T ratio of 20:1. Chromium-labelled T2 target cells were pre-incubated for 90 min with 1 μM of peptide. Chromium release was measured after 5 hr of incubation.

9-mer 154–162 as the most efficient peptide for sensitizing HLA-A*0201⁺ target cells for lysis by the gp100-reactive TIL 1200 line (Bakker *et al.*, 1995b). Recently, the gp100 154–162 9-mer has been eluted from the cell surface of HLA-A*0201⁺ melanoma cells, and was identified by tandem mass-spectroscopy, indicating that this peptide is indeed the nominal epitope endogenously presented in HLA-A*0201 (J. Skipper, personal communication). To identify amino-acid residues in the epitopes engaged in HLA-A*0201 binding and/or TCR interactions, epitope analogues were synthesized in which the native amino acid was replaced by an alanine residue. The substituted peptides were assayed for binding to HLA-A*0201 by means of a cell-bound HLA-A*0201-binding assay based on competition of a labelled reference peptide with the peptides of interest (Van der Burg *et al.*, 1995). For the gp100 154–162 epitope analogues, a decreased HLA-A*0201-binding capacity was observed for the alanine substitutions at positions 3 and 7 (Table I), indicating that these residues are important for HLA-A*0201 binding in addition to the anchor residues at positions 2 and 9. When the gp100 154–162 analogues were used at micromolar concentrations to sensitize HLA-A*0201⁺ target cells for lysis by gp100 154–162-specific CTL, we observed a drastic decrease in target-cell lysis for the alanine replacements at positions 5, 6 and 7 of the epitope (Table I). This demonstrates that the amino acids at these positions are critical residues within this epitope for interactions with the TCR.

Subsequently, the epitope analogues that induced reactivity at micromolar concentrations were titrated to evaluate their relative ability to sensitize T2 target cells for lysis by the relevant CTL (Fig. 1). In all cases the epitope analogues were similar or inferior to the wild-type epitope in their sensitizing capacity, except for the alanine substitution at position 8 of the gp100 154–162 epitope. This peptide was able to induce target-cell lysis by gp100-reactive CTL even at a 10-fold lower concentration.

*N-terminal anchor residue replacements in the gp100 154–162 epitope result in improved affinity for HLA-A*0201 and in enhanced T-cell recognition*

Since the gp100 154–162 epitope has a non-conventional N-terminal anchoring residue, we replaced this residue for the common HLA-A*0201 anchoring residues V, L, I or M (Ruppert *et al.*, 1993). Subsequently, we tested these peptides for HLA-A*0201 binding and their ability to sensitize target cells for lysis by anti-gp100 154–162 CTL. All anchor residue replacements in the gp100 154–162 epitope resulted in significantly improved binding to HLA-A*0201 (Table II). HLA-A*0201⁺ target cells loaded with these peptides at a concentration of 1 μM were recognized by the gp100 154–162 reactive CTL (Table II). Interestingly, these peptides were recognized by TIL 1200 when loaded on target cells at 10-fold lower concentrations compared to the wild-type peptide (Fig. 2), while the methionine-substituted peptide showed no difference. All peptides that were able to sensitize target cells for lysis by TIL 1200 at 10-fold lower concentrations, compared to wild-type, also displayed a higher HLA-A*0201-binding capacity. In addition to the N-terminal anchor substitutions, replacement of a polar residue for a hydrophobic residue adjacent to the C-terminal anchoring position also resulted in an epitope-analogue with an improved HLA-A*0201-binding capacity (KTWGQYWAV), apparently without affecting TCR recognition. These findings demonstrate that amino-acid substitutions at different positions within the native epitope can result in improved T-cell recognition.

Measurement of MHC class-I peptide complex dissociation rates demonstrated that the epitope analogues tested are at least as stable as wild-type epitopes (Fig. 3). All peptides that were able to enhance T-cell recognition showed a DT₅₀ (the time required for 50% of the complexes to decay) similar to or higher than that of the wild-type epitope (8 hr). Previously, it was shown that peptides with DT₅₀ values of ≥3 hours were immunogenic in HLA-A*0201/K^b transgenic mice (van der Burg *et al.*, 1996). Taken

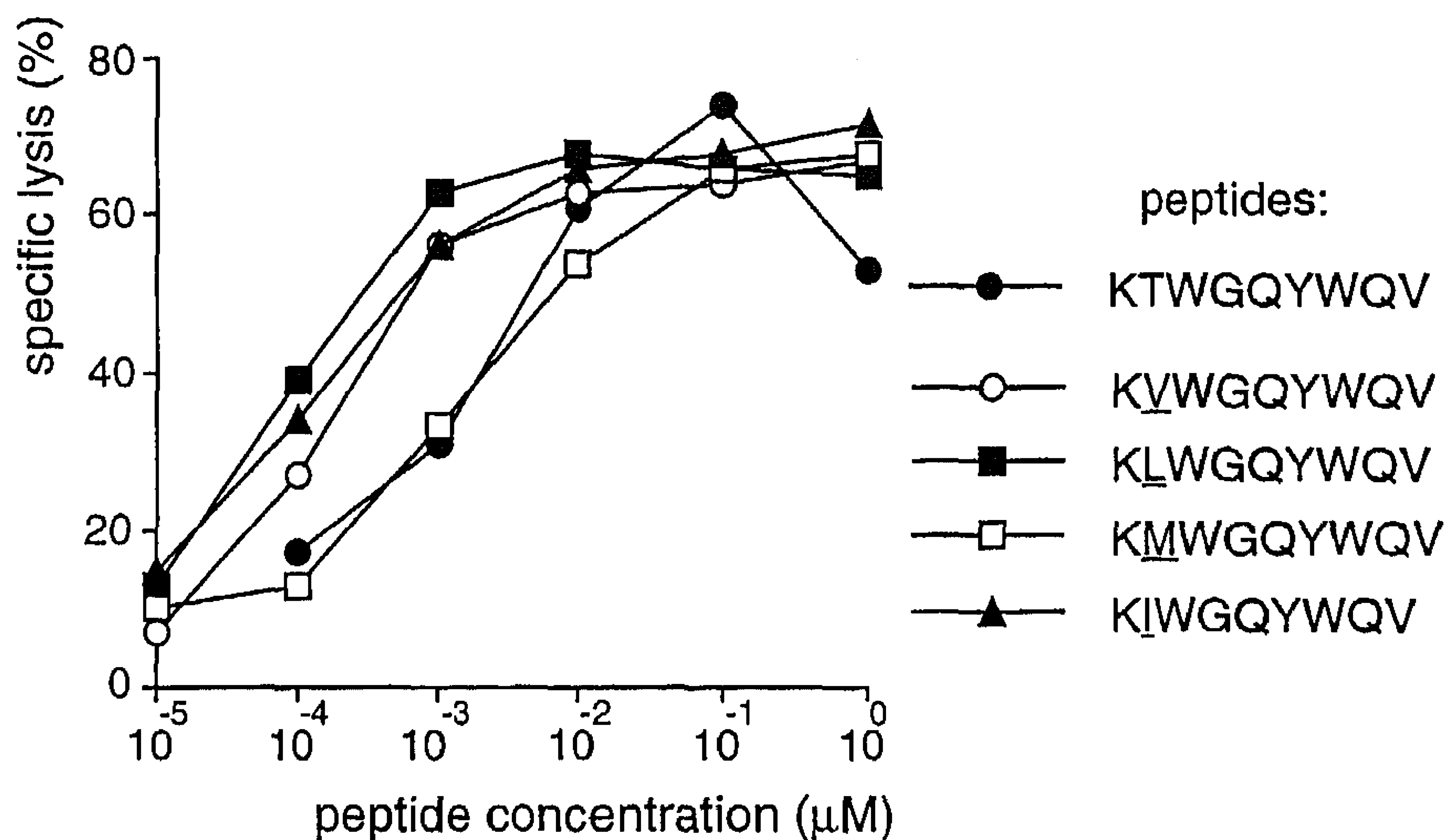


FIGURE 2 – Target-cell sensitization of gp100 154–162 N-terminal anchor-replacement epitopes. Chromium-release experiments were performed as in Figure 1. Gp100 154–162-reactive TIL 1200 lymphocytes were used to assay target-cell sensitization by the gp100 154–162 analogues at an effector-to-target ratio of 20:1.

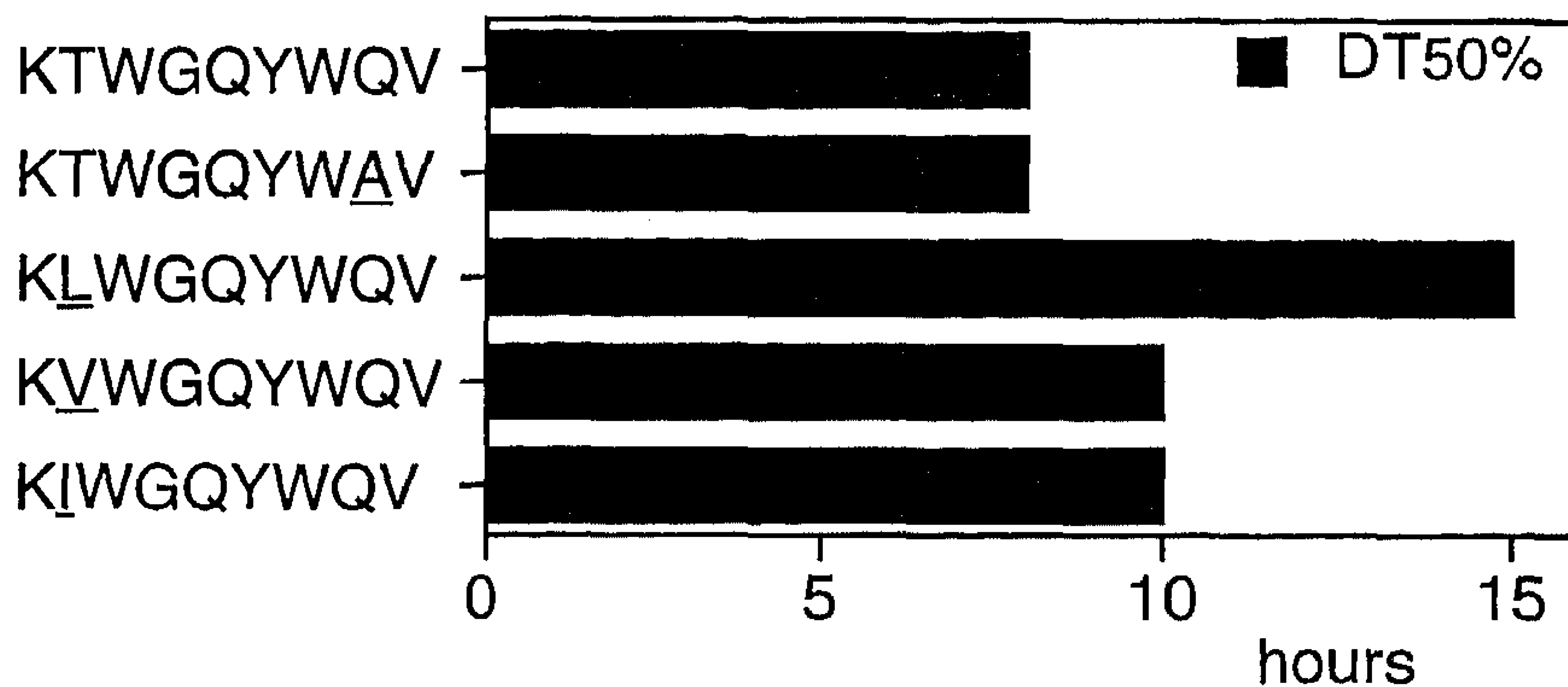


FIGURE 3 – HLA-A*0201-peptide complex stability of gp100 154–162 epitope analogues. The dissociation rate of HLA-A*0201-peptide complexes was measured using emetine pre-treated HLA-A*0201+ B-LCL. After mild-acid treatment, empty cell-surface HLA-A*0201 molecules were loaded at room temperature and the cells were then placed at a temperature of 37°C. The decay of cell-surface HLA-A*0201 molecules was analyzed by flow cytometry. The dissociation rate is depicted as the time required for 50% of the MHC class-I-peptide complexes to decay at 37°C.

together, these data indicate that the immunogenicity of the gp100 154–162 epitope analogues may be similar to, or higher than, that of wild-type gp100 154–162.

Immunogenicity of gp100 154–162 epitope analogues in HLA-A*0201/K^b transgenic mice

In order to determine the *in vivo* immunogenicity of the gp100 154–162 epitope analogues whose MHC class-I binding affinity and dissociation rate were measured, HLA-A*0201/K^b transgenic mice were vaccinated with the gp100 154–162 wild-type epitope, with the epitope analogues KTWGQYWAV, KVWGQYWQV, KLWGQYWQV or KIWGQYWQV, or with a control peptide (HBVcore 18–27; FLP_{SDDFPSV}). The generation of these transgenic mice (Vitiello *et al.*, 1991) and their use in analyzing *in vivo* immunogenicity have been described (Sette *et al.*, 1994). It has been shown that there exists an extensive overlap between the T-cell repertoires of HLA-A*0201/K^b transgenic mice and HLA-A*0201⁺ humans, supporting the use of HLA-transgenic mice to identify human CTL epitopes (Wentworth *et al.*, 1996). As shown in Figure 4, the gp100 154–162 epitope analogues KTWGQYWAV, KVWGQYWQV and KLWGQYWQV very efficiently induced a CTL response. To a lesser extent, the epitope analogue KIWGQYWQV and the wild-type gp100 154–162 were also able

to elicit a CTL response. Bulk CTL derived from mice vaccinated with the gp100 154–162 epitope analogues specifically lysed Jurkat A*0201/K^b cells loaded with both the peptide used for vaccination and the wild-type epitope. Interestingly, CTL bulk cultures raised against the epitope analogues all recognized target cells pulsed with the wild-type epitope as well as, or better than, target cells pulsed with epitope analogues used for vaccination. Thus, all gp100 154–162 epitope analogues tested were immunogenic in HLA-A*0201/K^b transgenic mice, and elicited CTL displaying cross-reactivity with the native gp100 154–162 epitope.

In vitro induction of gp100 154–162 epitope-analogue-specific human CTL displaying cross-reactivity with endogenously HLA-A*0201-presented wild-type gp100 154–162

Next we performed *in vitro* CTL induction assays to assess whether, within the T-cell repertoire of HLA-A*0201⁺, precursor T lymphocytes of healthy donors were present and capable of recognizing gp100 154–162 epitope analogues. In order to achieve this, we initiated cultures of peptide-loaded dendritic cells together with autologous responder T lymphocytes as described (Bakker *et al.*, 1995a). After several rounds of restimulation, responder T cells were tested for their cytotoxic activity (Fig. 5). All bulk CTL populations raised against the gp100 154–162 epitope analogues

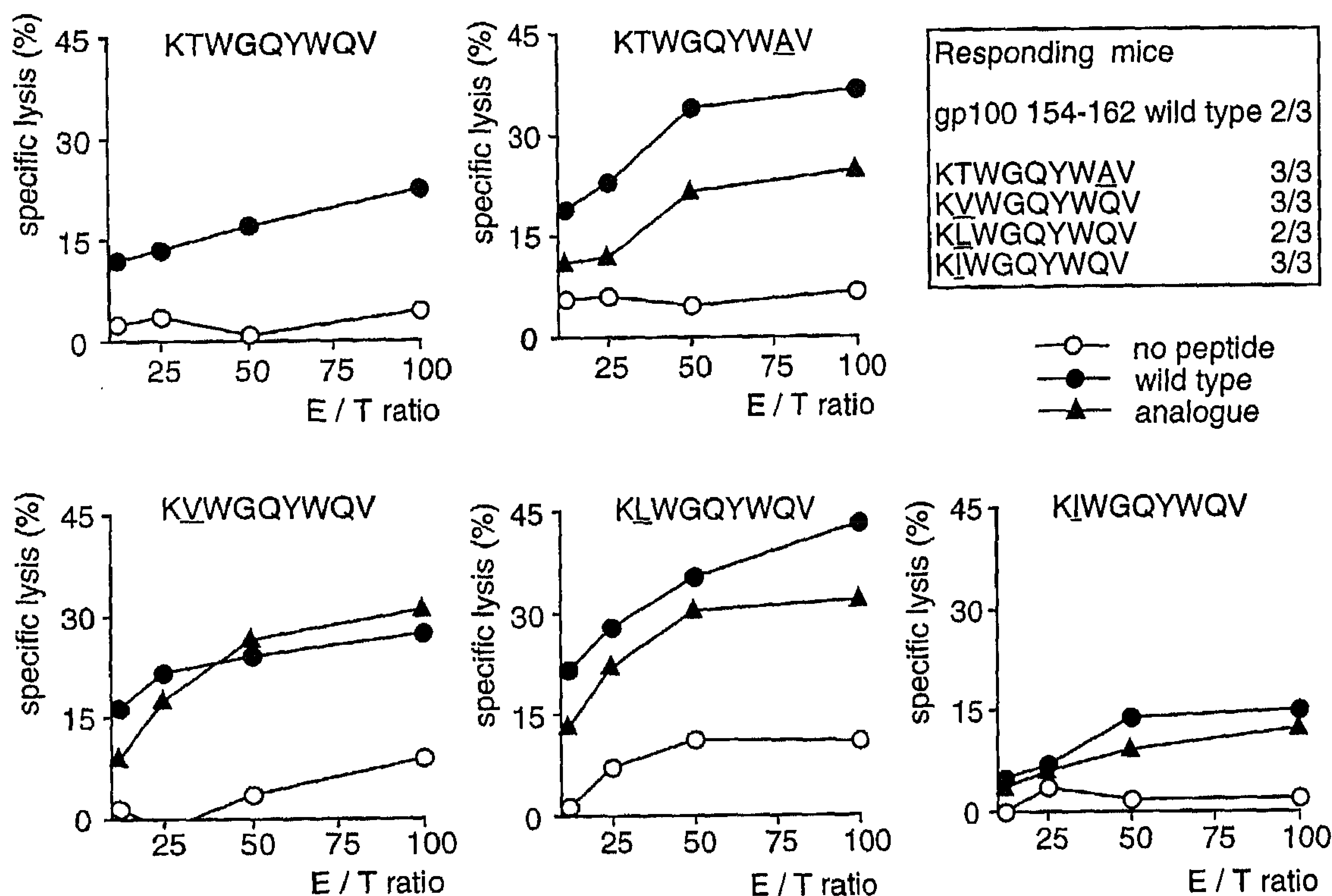


FIGURE 4 – Immunogenicity of gp100 154–162 epitope-analogues in HLA-A*0201/K^b transgenic mice. Bulk CTL obtained from immunized mice were tested for lytic activity using chromium-labelled Jurkat A2/K^b target cells that were pre-incubated with no peptide and 10 μ M wild-type gp100 154–162 or 10 μ M of the epitope analogue used to immunize the mice. For each peptide the mean specific lysis of bulk CTL of the responding mice is shown. Standard deviations never exceeded 15% of the mean value. One representative experiment out of 2 is shown.

KTWGQYWAV, KVGQYWQV, KLWGQYWQV and KIWGQYWQV efficiently lysed HLA-A*0201⁺ T2 target cells incubated with the peptides used for CTL induction. Only low background lysis was observed in the presence of an irrelevant peptide. In addition, these gp100 154–162 epitope-analogue-reactive CTL efficiently lysed T2 target cells incubated with wild-type gp100 154–162. To address the question of whether these CTL responder populations could also recognize endogenously processed and presented wild-type epitope, we performed chromium-release experiments using HLA-A*0201⁺ melanoma cell lines BLM and Mel 624 as targets. BLM cells have lost their expression of the gp100 antigen, at both the protein and the mRNA levels. As shown in Figure 6, all peptide-induced CTL cultures lysed the antigen-expressing Mel 624 cells, whereas no or background lysis was observed against antigen-negative BLM cells. TNF release by the anti-gp100 154–162 analogue CTL further demonstrated the reactivity of these CTL with endogenously presented wild-type gp100 154–162 (data not shown). These data show that the 4 different CTL cultures induced using gp100 154–162 epitope-analogue-loaded dendritic cells, all recognized the native gp100 154–162 epitope endogenously processed and presented by HLA-A*0201⁺ Mel 624 cells.

DISCUSSION

Since MHC class-I affinity and stability of the peptide-MHC complex are important parameters determining the immunogenicity of an MHC class-I-presented epitope, we explored the possibility of improving the capacity of a melanocyte differentiation antigen-derived epitope to bind to HLA-A*0201 without affecting interactions with the TCR. Detailed analysis of the gp100 154–162 epitope using alanine substitutions revealed that amino acids at positions 5 to 7 are critical residues for TCR recognition. These

data are in line with X-ray crystallography studies of the HLA-A*0201 molecule (Saper *et al.*, 1991), implying a role for the more permissive residues at positions 4 and 5 of the peptide oriented towards the outside of the MHC molecule, as prominent TCR contact sites. Our data confirm and extend these findings and demonstrate that, for HLA-A*0201, the amino acids at positions 6 and 7 of the gp100 154–162 epitope do not only interact with secondary pockets in the MHC peptide-binding cleft, but are also critical residues for TCR interaction (Ruppert *et al.*, 1993; Madden *et al.*, 1993).

The alanine substitution at position 8 in the gp100 154–162 epitope, KTWGQYWAV, resulted in a peptide that displayed increased HLA-A*0201 affinity. Moreover, this epitope analogue was recognized by gp100-reactive CTL at 10-fold lower concentrations compared to the native epitope. These data demonstrate that amino-acid substitutions at a non-anchor position can result in increased MHC class-I affinity and T-cell recognition. Similar findings were reported for the HLA-A*0201-restricted HIV-1 reverse transcriptase 476–484 epitope, in which substitutions at the first position of the peptide resulted in an increased stability of the peptide-MHC complex at the cell surface (Pogue *et al.*, 1995).

By N-terminal anchor replacements with V, L, M or I towards the HLA-A*0201 binding motifs, we set out to identify epitope analogues with improved HLA-A*0201-binding capacity that were still recognized by wild-type epitope-reactive CTL. For the gp100 154–162 epitope we obtained, in addition to the alanine-substituted analogue KTWGQYWAV, 3 anchor-substituted epitope analogues KVGQYWQV, KLWGQYWQV, and KIWGQYWQV, with improved HLA-A*0201-binding capacity that were recognized by anti-gp100 CTL at 10-fold lower concentrations compared to the wild-type epitope. *In vivo* immunization experiments using HLA-A*0201/K^b transgenic mice demonstrated that these epitope analogues were immunogenic, resulting in the induction of murine

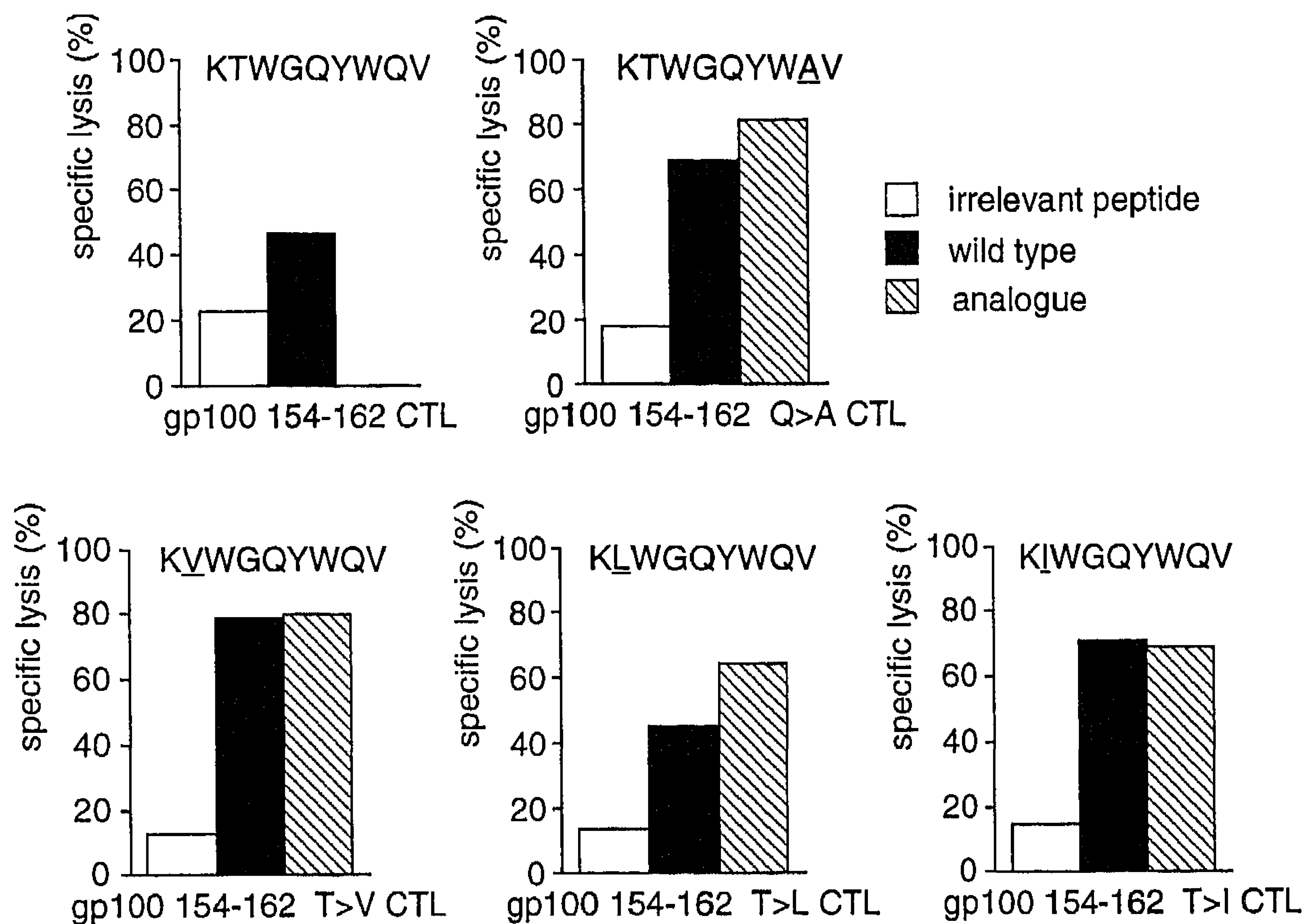


FIGURE 5 – Peptide-specific reactivity of *in vitro*-induced epitope-analogue-specific CTL cultures after 4 rounds of restimulation. Chromium-labelled HLA-A*0201⁺ T2 target cells were pre-incubated with 10 μ M of an irrelevant HLA-A*0201-binding peptide, 10 μ M wild-type gp100 154–162 or 10 μ M of the epitope analogue used for CTL induction. The different CTL cultures were added at an effector-to-target ratio of 20:1. One representative experiment out of 2 is shown.

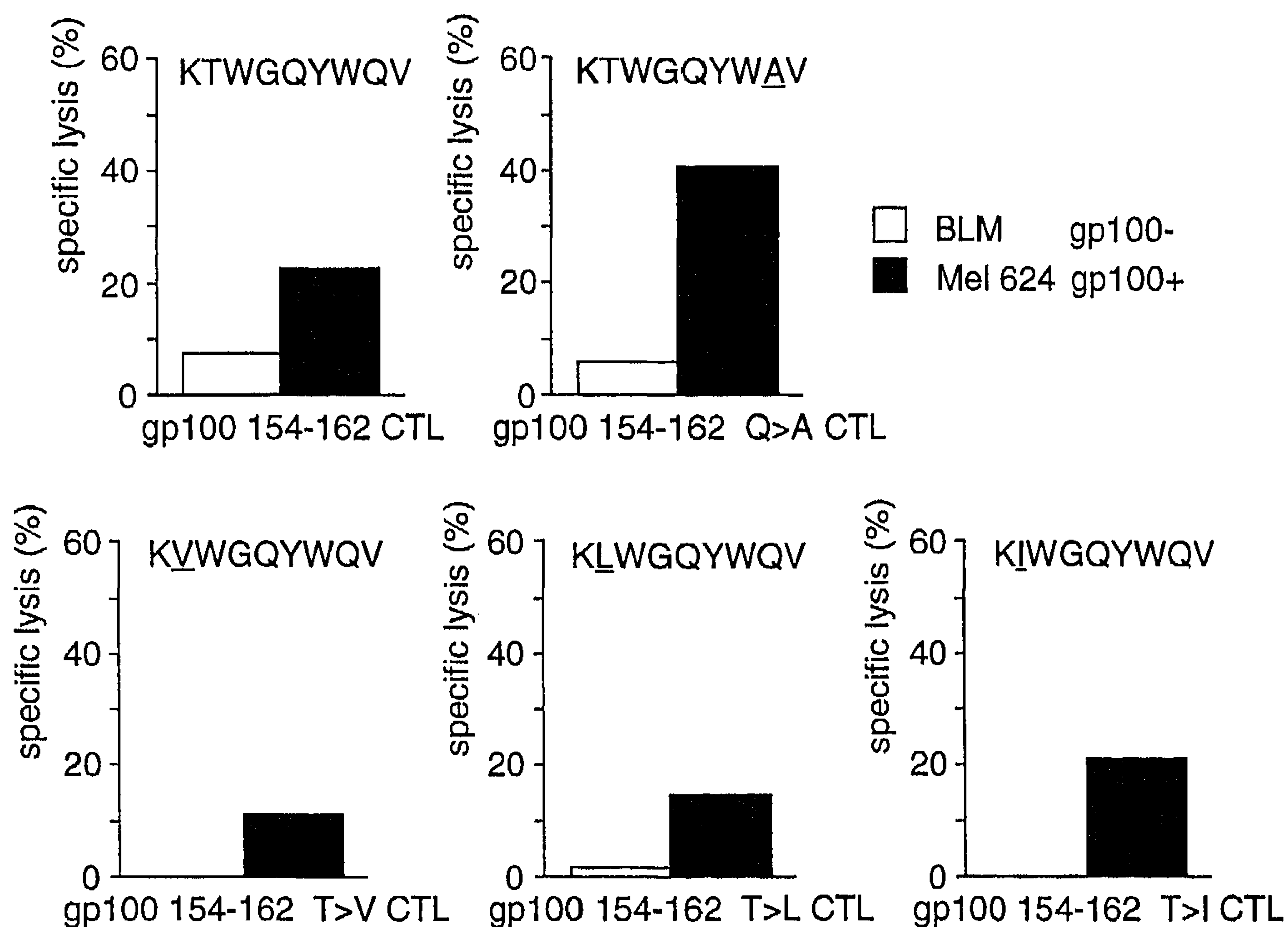


FIGURE 6 – Epitope-analogue-induced CTL cultures obtained after 4 restimulations specifically lysed melanoma cells endogenously presenting the wild-type epitope. Chromium-labelled HLA-A2.1⁺ BLM and Mel 624 melanoma cells were used as target cells. BLM cells lack expression of gp100. The different CTL cultures were added at an effector-to-target ratio of 20:1. One representative experiment out of 2 is shown.

CTL reactive with both the epitope analogues and the native epitope. The immunogenicity of the epitope analogues was expected since the stability of the peptide-MHC complex of both the epitope analogues and the native epitope was comparably high. Therefore, our results confirm previous data demonstrating that peptides with low MHC class-I-peptide complex dissociation rates can effectively induce CTL responses (van der Burg *et al.*, 1996).

In vitro CTL induction experiments using donor-derived PBL demonstrated that epitope-analogue-specific CTL could be obtained displaying cross-reactivity with tumor cells endogenously presenting the wild-type epitope. In addition to T lymphocytes reactive with the wild-type epitope, the T-cell repertoire of healthy donors apparently also contains T cells reactive with the gp100 154–162 epitope analogues. Analysis of TCR usage of cloned CTL reactive with the different gp100 154–162 epitope analogues and with wild-type gp100 154–162, which is currently under investigation, will be informative of the spectrum of the T-cell repertoire that can be used to induce CTL reactivity towards the wild-type epitope. Previously, it was observed that the repertoire of CTL directed against the MAGE-1 161–169 antigenic tumor peptide (HLA-A*0101) in a melanoma patient can be diverse in terms of both antigen recognition and TCR usage (Romero *et al.*, 1995). Analyses of TCR repertoires specific for a variety of antigenic peptides presented in MHC class-I molecules have shown that they can be either relatively homogeneous (Kalams *et al.*, 1994; Lehner *et al.*, 1995) or highly diverse (Taylor *et al.*, 1990; Cole *et al.*, 1994). Thus far it is not clear what determines the degree of diversity in the TCR repertoires. It has been proposed that limited TCR repertoires represent “public” CTL specificities, that can be reproducibly isolated from different individuals in experimental models (Cibotti *et al.*, 1994). In addition to these dominant public repertoires, “private” TCR repertoires can exist that are more diverse and that differ for each individual. It is tempting to speculate that, in the case of self-antigens, public CTL specificities are rendered non-functional due to tolerizing mechanisms while the private TCR repertoire is unaffected. With respect to immunotherapy of cancer,

potential activation of multiple specificities in the private T-cell repertoire against an antigenic tumor epitope using epitope analogues may stimulate immunoreactivity after vaccination, resulting in an anti-tumor response.

In summary, we describe here the identification of epitope analogues of the gp100 154–162 epitope with potentially improved immunogenicity, that were obtained by amino-acid substitutions in both anchor and non-anchor positions in the native epitope. In addition to the gp100 154–162 epitope, we explored this methodology to select analogues for the low HLA-A*0201-affinity MART-1 27–35 epitope (data not shown). Recently, it was shown for 2 other gp100-derived epitopes, located at positions 209–217 and 280–288, that amino-acid substitutions at anchoring positions may result in epitope analogues with improved immunogenicity (Parkhurst *et al.*, 1996). Using these epitope analogues, the authors demonstrated an enhanced induction of melanoma-reactive CTL from HLA-A*0201⁺ melanoma patients compared to the native epitopes. Employment of “improved” epitopes in immunotherapy protocols may increase the amount of peptide-MHC complexes at the cell surface of antigen-presenting cells *in vivo*, and may thus result in enhanced priming of antigen-specific CTL. Apart from their potential in cancer immunotherapy, the use of epitope analogues with improved immunogenicity may contribute to the development of CTL-epitope-based vaccines in chronic viral disease.

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